Respiratory ATP synthesis as drug target for combating tuberculosis

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Chapter 1

General introduction

1.1 Introduction

Mycobacterium tuberculosis accounts for nearly 2 million deaths per year and is the predominant cause of death in HIV patients [Check, 2007]. At present, tuberculosis is treated with a cocktail of antibiotics for at least six months. In the first two months, daily dosages of the first-line drugs isoniazid, rifampin, pyrazinamide, and ethambutol are applied, followed by four months treatment with isoniazid and rifampin alone [Mitchison, 2005]. This very long treatment is due to populations of *M. tuberculosis* in a metabolic resting state, referred to as dormancy or latency. In this resting state the bacteria can reside within human macrophages for many years, without causing active disease. Dormant mycobacteria display increased cell envelope thickness and significantly remodeled metabolic routes as compared with the replicating state, e.g. nucleic acid synthesis and protein synthesis are strongly down-regulated in this non-replicating state [Wayne & Sohaskey, 2001]. Currently used antibiotics have poor activity against non-replicating bacilli [Gomez & McKinney, 2004] due to low cell envelope penetration and decreased importance of the metabolic pathways targeted by most drugs.

Patients often experience difficulties following a long-term treatment regimen, resulting in low treatment compliance, which in turn directly contributes to the emergence of multidrug- and extensively drug-resistant strains of *M. tuberculosis* [Check, 2007, Dye, 2009]. Infections with these drug-resistant mycobacterial strains require an even longer chemotherapy of 6-24 months [Gandhi

et al., 2010, Koul *et al.*, 2011].

For improvement of current tuberculosis treatment development of novel antimycobacterial compounds as well as the discovery, validation and characterization of new target proteins are of key importance [Bald & Koul, 2010, Dye & Williams, 2010, Koul *et al.*, 2011, Russel *et al.*, 2010].

1.1.1 Energy metabolism as target pathway for antibacterials

The antibacterial pyrazinamide, in clinical use as first-line tuberculosis drug since the early eighties [Mitchison, 1985], provided the first indication that energy metabolism could be an attractive target for drug development. Pyrazinamide is hydrolyzed within the mycobacterial cell, yielding the active entity, pyrazinoic acid. Pyrazinoic acid is thought to act as a weak acid, interfering with the bacterial proton motive force [Zhang *et al.*, 2003].

In 2005, two enzymes complexes of respiratory ATP synthesis were reported as new targets of antituberculosis drugs: NADH dehydrogenase [Weinstein *et al.*, 2005] and ATP synthase [Andries *et al.*, 2005]. The phenothiazines and phenothiazine analogues inhibit the type II NADH dehydrogenase in *M. tuberculosis*. Phenothiazine inhibition of NADH oxidation strongly suppresses oxygen consumption and ATP synthesis activity by *M. tuberculosis* membranes [Boshoff *et al.*, 2004, Weinstein *et al.*, 2005, Yano *et al.*, 2006]. Moreover, preventing NADH oxidation can increase the NADH/NAD+ ratio, leading to disturbed cellular redox balance [Rao *et al.*, 2008].

Diarylquinolines inhibit ATP synthesis and are active on drug-sensitive and drug-resistant *M. tuberculosis* strains [Andries *et al.*, 2005, Diacon *et al.*, 2009, Huitric *et al.*, 2007, Koul *et al.*, 2007]. Diarylquinoline lead compound TMC207 is bactericidal against replicating and non-replicating mycobacteria [Koul *et al.*, 2007, Koul *et al.*, 2008, Rao *et al.*, 2008]. Interestingly, TMC207 shows a delayed bactericidal action, with little effect in the first days of drug addition and onset of bacterial killing after 3-4 days [Lounis *et al.*, 2008, Rustomjee *et al.*, 2008]. *Mycobacterium leprae* and *Mycobacterium ulcerans*, the causative agents of leprosy and buruli ulcers in humans, are also effectively killed by TMC207 [Ji *et al.*, 2006a, Ji *et al.*, 2006b]. In a murine model of tuberculosis TMC207 as monotherapy proved at least as active as the combination of the first-line antibiotics rifampin, isoniazid and pyrazinamide [Andries *et al.*, 2005]. TM207 showed strong synergy with pyrazinamide, this combination therapy renders almost all infected mice culture-negative after two months of treatment [Andries *et al.*, 2005, Ibrahim *et al.*, 2007, Lounis *et al.*, 2006]. This indicates that a

therapy including TMC207 and pyrazinamide could have the potential to significantly shorten tuberculosis treatment. The long half-life of TMC207 [Andries *et al.*, 2005] and the low proportion of resistant development to TMC207 [Andries *et al.*, 2005, Huitric *et al.*, 2010] are favorable characteristics for usage in intermittent therapy of tuberculosis. The combination of TMC207, rifapentine and pyrazinamide administered intermittently was found more active than a daily regimen of the current first-line therapy isoniazid, rifampin and pyrazinamide [Veziris *et al.*, 2009], raising the possibility of developing a new fully intermittent short-course regimen for the therapy of tuberculosis.

1.1.2 The mycobacterial respiratory chain

Electrons can enter the respiratory chain via oxidation of NADH using NADH dehydrogenase. Most mycobacteria possess two types of NADH dehydrogenases in their respiratory chain. The proton-translocating type I NADH dehydrogenase apparently is not essential for growth of *M. tuberculosis* [Rao *et al.*, 2008, Sassetti *et al.*, 2003]. Alternatively, a non-proton-translocating type II NADH dehydrogenase serves as the primary NADH dehydrogenase for NADH oxidation and utilizes menaquinone as an electron acceptor (Fig. 1.1). After accepting electrons from the type II NADH dehydrogenase, menaquinol can be oxidized either by cytochrome bc_1 reductase (subunits QcrA-C), which then transfers the electrons to an aa_3-type cytochrome c oxidase (subunits CtaC-F) [Matsoso *et al.*, 2005], or directly by cytochrome bd oxidase (subunits CydA-B) [Kana *et al.*, 2001] (Fig. 1.1, for a review see [Cox & Cook, 2007]). The route via the cytochrome bc_1 reductase-cytochrome c oxidase supercomplex is bioenergetically favorable as electron transport and the reduction of O_2 at the aa_3-type cytochrome is coupled with proton translocation across the membrane. However, cytochrome bd oxidase displays a higher affinity for oxygen and is thus used under O_2-limiting conditions [Kana *et al.*, 2001], whereas the cytochrome aa_3-type enzyme is the predominant terminal electron acceptor during aerobic growth [Shi *et al.*, 2005]. ATP synthase utilizes the proton motive force across the cytoplasmatic membrane for synthesis of adenosine-5′-triphosphate (ATP) from adenosine phosphate (ADP) and inorganic phosphate (P_i). The enzyme consists of a cytosolic F_1 part (subunits $\alpha_3\beta_3\gamma\delta\varepsilon$) and the membrane-embedded F_0 entity (subunits ab_2c_{10-15}), which are responsible for catalysis of ATP synthesis and for proton flow, repectively (Fig. 1.1). ATP synthase is evolutionarily strongly conserved among prokaryotes and eukaryotes.

In contrast to several other bacteria, which can produce sufficient ATP by substrate-level phosphorylation and tolerate deletion of ATP synthase [Jensen

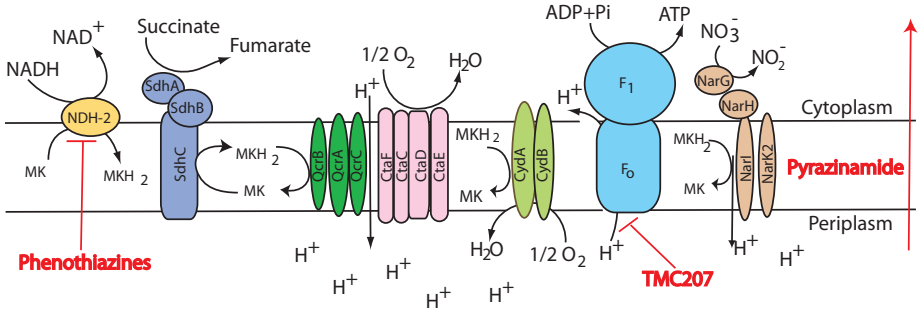

Figure 1.1: Schematic view of the mycobacterial respiratory chain. The menaquinone (MK) pool can be reduced by either NDH-2 (yellow) or via succinate (dark blue) and oxidized by either a cytochrome bc_1 (dark green)/cytochrome aa_3 (pink) super-complex or by the cytochrome bd oxidase (light green). The proton motive force is used by ATP synthase (light blue) for the production of ATP. The nitrate reductase and transporter system is shaded in brown. The type-I-proton-translocating NADH dehydrogenase, which is not essential for growth, is not shown. Small-molecule compounds, which block respiratory ATP production and act bactericidally on replicating and dormant mycobacteria, are depicted in red (adapted from [Bald & Koul, 2010].

& Michelsen, 1992], ATP synthase has been proven to be essential for optimal growth in *M. tuberculosis* [Sassetti *et al.*, 2003] and for growth on fermentable and nonfermentable carbon sources in *Mycobacterium smegmatis* [Tran & Cook, 2005]. This enzyme in principle can also function in the reverse direction as a proton pump to maintain a proton motive force under condicitons of low oxygen tension or low nutrient availability [von Ballmoos *et al.*, 2009]. However, ATP synthase from several bacteria displays only very limited ATP hydrolysis activity, for example in *Paracoccus denitrificans* [Harris *et al.*, 1977], *Bacillus subtilis* [Hicks *et al.*, 1994] *Thermus thermophilus* [Nakano *et al.*, 2008] and *Mycobacterium phlei* [Higashi *et al.*, 1975].

TMC207 specifically binds to subunit c of ATP synthase [Koul *et al.*, 2007]. During enzymatic catalysis, this oligomeric subunit, together with subunits ε and γ, rotates relative to subunits $\alpha_3\beta_3\delta ab$ and in this way couples proton flow to the synthesis of ATP [Boyer, 1993, Junge *et al.*, 1997]. Protons enter from the periplasmic space via an entry channel in subunit a and are then transferred to an essential acidic residue in the membranespanning part of subunit c (Fig. 1.2). After a close to 360° rotation of the cylindrical subunit c oligomer relative to subunit a, the protons are released on the cytosolic side of the membrane via

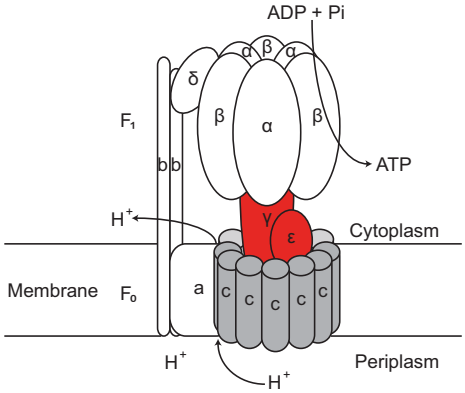

Figure 1.2: Schematic view of ATP synthase and proton flow during ATP synthesis. During the synthesis of ATP, the oligomeric subunit c (dark gray), together with subunits γ and ε (red), rotates relative to the other subunits of the ATP synthase complex.

an exit channel in subunit a [Diez *et al.*, 2004, Vik & Antonio, 1994]. Mutations in subunit c conferring resistance to TMC207 suggest a binding site in the central region of subunit c, close to the essential acidic residue [Andries *et al.*, 2005, Huitric *et al.*, 2007, Huitric *et al.*, 2010, Koul *et al.*, 2007, Petrella *et al.*, 2006].

1.2 Outline of the thesis

Chapter 2

describes the investigation of the selectivity of TMC207 towards mycobacterial ATP synthase compared with that towards mitochondrial ATP synthase. In a sub-cellular mycobacterial membrane assay and human mitochondria the effect of TMC207 on ATP synthesis was determined. The study reveals that human mitochondrial ATP synthase displayed more than 20,000-fold lower sensitivity for TMC207 compared to that of mycobacterial ATP synthase, indicating that the compound is highly specific for mycobacteria and unlikely to be toxic for mammalian cells. Our results illustrate that ATP synthase is a promising drug target, despite the fact that the enzyme is conserved among prokaryotes and eukaryotes.

Chapter 3

describes the characterization of the physiological role of ATP synthase in slow-growing mycobacterial strains. ATP synthase in inverted membrane vesicles from the slow-growing model strain *M. bovis* BCG is active in ATP synthesis, but blocked in ATP hydrolysis direction. These results suggest that ATP synthase is needed for synthesis of ATP, not for maintenance of the proton motive force. For the development of new antimycobacterial drugs acting on ATP synthase, screening for ATP synthesis inhibitors, but most likely not for ATP hydrolysis blockers, can be regarded as a promising strategy.

Chapter 4

describes the mode of binding between TMC207 and mycobacterial ATP synthase. Factors that can potentially influence binding between the compound and its target, such as the proton motive force, the pH value and buffer ionic strength, were investigated using biochemical assays and binding studies. Independent of environmental conditions such as the local pH and the proton motive force, TMC207 displays high affinity for its target. The drug binds to a defined binding site in ATP synthase, most likely blocking conformational changes associated with proton flow. These properties, combined with the essentiality of the target, may in part explain the exceptional ability of this compound to efficiently kill mycobacteria in different microenvironments.

Chapter 5

describes the metabolic response of a slow-growing mycobacterium, *M. bovis* BCG, to inhibition of ATP synthase by TMC207. Elucidating the metabolic response to antibiotic action can reveal points of vulnerability in bacterial metabolism. In the presence of TMC207 the major ATP consuming pathways, such as nucleotide synthesis, DNA synthesis and protein synthesis, were downregulated, indicating a general response to conserve cellular energy. Activation of the glyoxylate cycle and upregulation of enzymes of β-oxidation indicates a shift to lipid metabolism. The observed downregulation of ATP consumption may in part explain the delayed bactericidal effect of TMC207.

Chapter 6

describes the isolation of ATP synthase from a slow-growing mycobacterial strain. The isolated detergent-solubilised enzyme displays low ATP hydrolysis

activity and is sensitive to N, N'-dicyclohexyl-carbodiimide. As mycobacterial ATP synthase has recently been validated as target of new class of candidate drugs against tuberculosis, the diarylquinolines, availability of isolated ATP synthase may help to investigate interaction between drug and target. Moreover, the isolated enzyme may be used to shed light on the unusual subunit composition of mycobacterial ATP synthase, as suggested by genomic data.

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